METABOLIC PATHWAYS OF HEXACHLOROBENZENE (HCB), QUINTOZENE (PCNB) AND PENTACHLOROPHENOL (PCP) BY A NEWLY ISOLATED STRAIN *Nocardiodes* sp. PD653 UNDER AEROBIC CONDITIONS

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Abstract

A novel bacterial strain PD653 was isolated from a quintozene (PCNB)-degrading bacterial consortium enriched by original soil-charcoal perfusion system with a contaminated upland soil (Ibaraki, Japan). The isolate was capable of degrading PCNB, hexachlorobenzene (HCB) and pentachlorophenol (PCP) as the solo carbon source in an aquatic batch culture. The strain PD653 was identified as that belonging to *Nocardioides* sp. on the basis of comparative morphology, physiological characteristics and comparison of the 16S rRNA gene sequence. Approximately 40% of [U-ring-^{14}C] HCB was mineralized by PD653 during 2-week cultivation at 30°C. PCNB and HCB are converted into PCP. Cl^- and NO_2^- were produced in the culture with HCB and PCNB respectively, therefore we concluded that strain PD653 can remove chlorine and nitro groups via oxidative pathway. Monooxygenase might be involved in this reaction. PD653 is the first wild bacteria that can mineralize HCB aerobically.

Introduction

HCB (C_6Cl_6) is one of the most persistent environmental pollutants, and bioaccumulates in the environment, in animals, and in humans. Therefore, HCB was listed as one of the 12 persistent organic pollutants (POPs) in the Stockholm Convention. Microbial degradation is a promising effective way to remediate environmental pollutants, including POPs. Several studies have reported reductive dechlorination[1-5] and biodegradation with genetically engineered bacterial cells.[6] However, pure culture capable of mineralizing HCB aerobically has not been isolated yet. In our previous study, we have reported the isolation of PCNB-degrading bacteria, strain PD653. Interestingly, PD653 utilized HCB as solo carbon source.[7] In this study, detailed taxonomic studies were carried out. Furthermore, we report the characterization of initial metabolite caused by oxidative removal of the nitro and chlorine groups from PCNB and HCB, respectively.

Materials and Methods

1) Enrichment culture by using the soil-charcoal perfusion system [8, 9]

Enrichment of PCNB-degrading bacteria was performed by the soil-charcoal perfusion system. A soil sample, to which PCNB had been annually applied for more than 5 years, was taken from a cabbage field (Ibaraki, Japan) at a depth of 0-20 cm. Table 1 shows physical and chemical characteristics of the soil sample. The soil sample (40g, dry weight) was mixed with Charcoal A (2 g, grain size 5 to 10 mm, specific surface area 100 m^2/ g, pH 7.8) as a microhabitat and an adsorbent of PCNB. Enrichment culture was carried out under dark conditions at 25°C by circulating 300 ml mineral salt medium (MM) containing 5 mg/L of PCNB through the soil-charcoal mixture in a perfusion apparatus. The perfusion rate of the medium was adequately controlled by a portable air pump and smooth leaching was maintained. The medium was replaced every week. Aliquots of culture fluids were centrifuged at 12,000 rpm for 10 min. Concentration of chloride ion in the supernatant was measured by an ion chromatography. For determining the PCNB concentration, the supernatant (5 ml) was passed through a Sep-Pak C18 cartridge, which was pre-conditioned by washing with acetone (5 ml), methanol (5 ml) and distilled water (8 ml), with a Waters vacuum manifold. The “concentrated” cartridge was then dried over the vacuum manifold for approximately 20 min and subsequently eluted with acetone (5 ml). The eluate was dried and re-dissolved with 1 ml acetone for an electron capturer detector-gas chromatograph (ECD-GC). Following the detection of PCNB degradation, the charcoal (0.25 g) was transferred to another apparatus with new Charcoal A (7.5 g). Circulation was then continued for 6 weeks for further purification and enrichment.

Table 1 physical and chemical characteristics of the soil sample

Moisture Content[a](%)	pH (H_2O)	pH (KCl)	T-C (%)	T-N (%)	C/N ratio
35.8	6.3	5.7	4.24	0.36	11.8

[a] After sampling and sieving

2) Isolation of PCNB-degrading bacteria

The charcoal (1 g), on which PCNB-degrading bacteria had been enriched, was crushed and suspended in 50 mM phosphate buffer (pH 7.0). The suspension was diluted with the same buffer and was inoculated on the MM agar plate containing 50 mg/L of PCNB. Following successive incubation at 25°C for 3 weeks, colonies showing a clear zone on the plate were isolated and subcultured on the same agar medium. Further purification of a PCNB-degrading bacterium was conducted on R2Aagar plates at 30°C.

3) Identification of the isolated PCNB-degrading bacteria

The isolated strain, PD653, was characterized on the basis of comparative morphology, physiology and comparison of the 16S rRNA sequences. The known primers named fD1, fD2, rP1, rP2 and rD1 was used for 16S rRNA gene amplification, and the following 5'-Texas Red-labeled primer was used for the cycle sequencing reaction: fD1, rD1, 341f, 534r, 799f (5'-CAAACAGGATTAGATACCC-3'), 907f, 907r and 1223r.

4) Biotransformation experiment

Cells of strain PD653 were grown on R2A medium until they reached an optical density (OD_{600}) of about 1.2. After harvesting the cells by centrifugation (4,000 × rpm, 20 min), the cell pellets were washed twice with a 20 mM phosphate buffer (pH 7.0) and suspended in the same buffer.

Prior to the experiments, HCB and PCNB were dissolved in an acetone solution at a concentration of 500 mg/L. An appropriate aliquot of this stock solution was then added to a sterilized Erlenmeyer flask, and the solvent mixture was evaporated in the ambient atmosphere. For turnover experiments with resting cells, 10 ml portions of a cell suspension (OD_{600}=1.0) were added to 50 ml Erlenmeyer flasks containing PCNB and HCB (6 mg/L).

After shaking for several times in a rotary shaker at 22°C, 1 ml of 1 M HCl and 10 ml of CH_3CN were added to stop the reaction. Aliquots were withdrawn (1.0 ml), then the sample was centrifuged (13,000×g, 10 min), and 20 μl of the supernatant was then subjected to high-performance liquid chromatography (HPLC) analysis.

Results and Discussion

In the perfusion apparatus, the disappearance of PCNB and generation of chloride ion were detected after 1 week of circulation. Following 3 weeks of circulation, the charcoal was removed and inoculated to the secondary enrichment culture. The dissipation rate of PCNB and the generation rate of chloride ion increased after 3 weeks of circulation in the secondary culture. The charcoal, in which the PCNB-degrading bacteria were enriched, was harvested following 6 weeks of cultivation.

A bacterial colony showing a clear zone on MM agar containing 50 mg/L of PCNB was isolated from the enriched charcoal and was named PD3. However, PD3 was a mixed culture consisting of four bacterial species. In order to purify PCNB-degrading bacterium, the PD3 culture was plated onto R2A agar plate and incubated for 2 weeks. The ability of the individual colonies to degrade PCNB was examined in MM containing 5 mg/L of PCNB (20 ml) for 10 days at 25°C, 120 rpm, and the PCNB-degrading isolate, strain PD653, was obtained. Strain PD653 could degrade PCNB with liberation of chloride ion and nitrite ion. After 10 days of cultivation, strain PD653 completely degraded 1 mg/L of HCB and PCP as well as PCNB; however, it did not degrade chlorothalonil and dichlobenil.

The isolated strain PD653 belongs to a species of gram-negative, catalase-positive, oxidase-negative, non-spore-forming and non-motile rods (0.7-0.8 x 1.0-1.2μm in size), which form pale yellow circular colonies. The GC content of the strain was 70.8%. The sequence analysis of the bacterium determined 1,487 bp of the 16S rRNA gene. On comparison with the sequences available in the GenBank database, the gene sequence had 97.1%

sequence identity with *Nocardioides* sp. OS4, 96.9% with *Nocardioides* sp. JS884 and 96.6% with *Nocardioides aquiterrae* GW-9. Based on these results, strain PD653 is assigned to a novel species in the genus *Nocardiodes.*
HPLC chromatograms derived from cell incubation with PCNB and HCB were shown in Fig.1. PCNB and HCB were converted into pentachlorophenol (PCP). This metabolite was identified on the basis of its HPLC retention time and UV spectrum, which were found to be identical to those of an authentic sample. Cl^- and NO_2^- were produced in the culture with HCB and PCNB, respectively; therefore we concluded that strain PD653 can remove chlorine and nitro groups via oxidative pathway. Monooxygenase might be involved in this reaction. Including our previous study [7], the metabolic pathways of HCB, PCNB and PCP by strain PD653 was proposed in Fig.2
The microbial degradation of nitroaromatic compounds has been extensively investigated and the removal of the nitro group is carried out via oxidative pathways that initiate with monooxygenases[10] or dioxygenases[11], or a partial reductive pathway that initiates with nitroreductase.[12] The chlorinated nitroaromatic compounds are more resistant to microbial degradation due to the simultaneouse existence of chlorine and nitro groups, and thus the knowledge of its microbial degradation is very limited. Previous studies revealed that reductive dehalogenation and partial reduction of nitro groups might be involved in the initial steps during chlorinated nitroaromatic compound degradation. In the present study, strain PD653 converted PCNB to PCP via oxidative removal of nitro group. This is the novel reaction on the microbial degradation of heavily chlorinated nitroaromatic compounds.
Conversion of HCB to PCP was reported by using the mutant of cytochrome P-450cam (CYP101) from *Pseudomonas putida* by genetic engineering.[13] However, there is no report of the aerobic conversion of HCB to PCP by natural microorganism.
Aerobic micro-organisms degrade chlorinated aromatic compounds via initial attack by nonheme iron dioxygenases to form the *cis*-dihydrodiol. After re-aromatization to chlorocatechols, catechol 1,2-dioxygenases cleave the aromatic ring.[14, 15] Both of these dioxygenases appear to require certain ring positions to remain unsubstituted.[16, 17] No strictly aerobic organism capable of degrading HCB or other heavily chlorinated benznes has been isolated. Polychlorinated phenols are readily mineralized by micro-organisms. Heavily chlorinated phenols oxidized to chlorohydroquinones, and reactive chlorohydroquinones are subsequently dehalogenated by hydrolytic or reductive mechanisms. Therefore, strain PD653 capable of converting heavily chlorinated benzenes to chlorinated phenols has the great advantage in development of future environmental remediation technology.

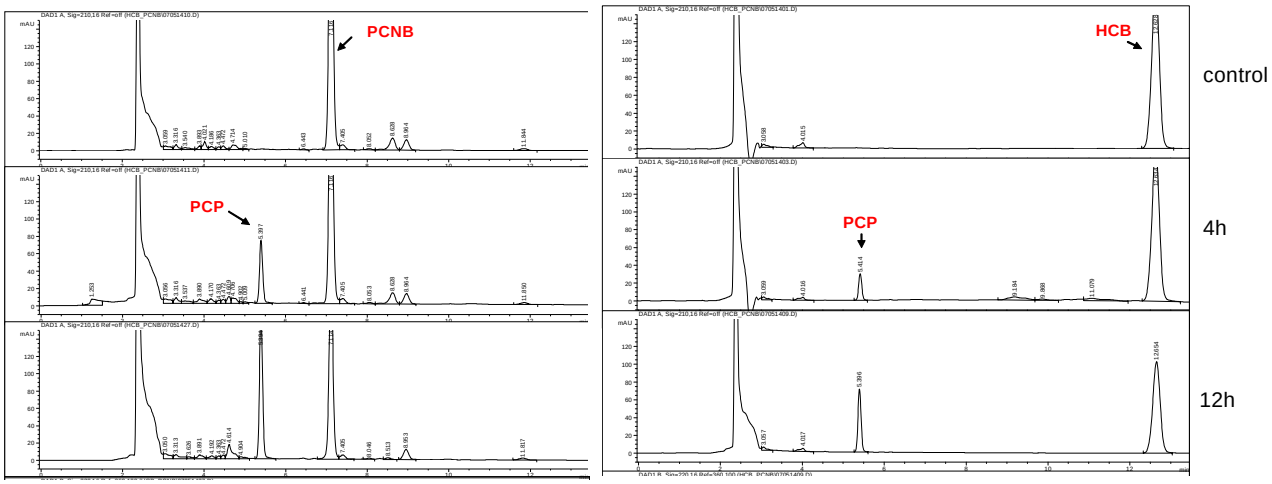

Fig.1 HPLC profiles of aliquots from resting cell culture 4 h and 12 h after addition of 6 mg/L of PCNB or HCB

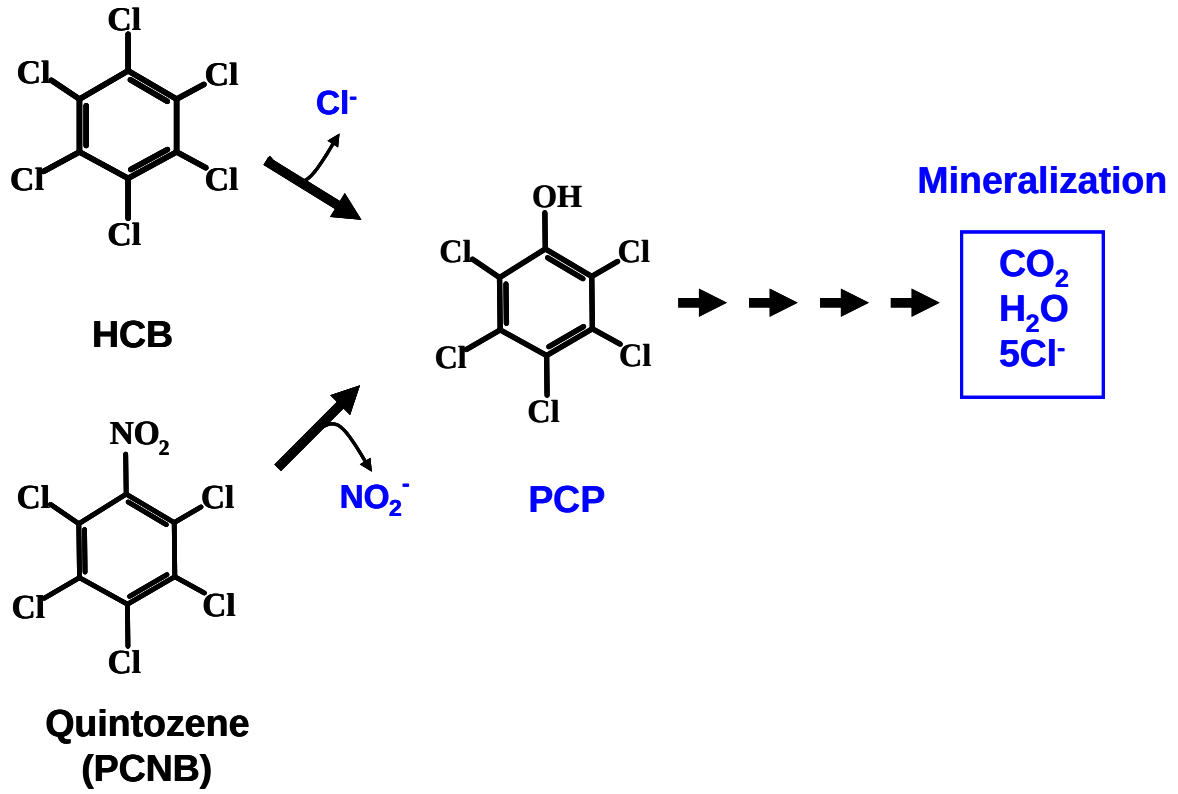

Fig.2 Proposed metabolic pathways of HCB, PCNB and PCP by PD653 under strict aerobic conditions

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